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Enhanced microbial bile acid deconjugation and impaired ileal uptake in pregnancy repress intestinal regulation of bile acid synthesis

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List of abbreviations

FXR: farnesoid X receptor; RXR: retinoid X receptor; IBABP: ileal bile acid binding protein; OST: organic solute transporter; FGF: fibroblast growth factor; SHP: small heterodimer partner; ASBT: apical sodium dependent bile acid transporter; GLP1: glucagon-like peptide 1; MCA: muricholic acid; C4: 7α-hydroxy-4-cholesten-3-one; CDCA: chenodeoxycholic acid; PM5S: epiallopregnanolone sulfate; ELISA: enzyme-linked immunosorbent assay; HPLC: high performance liquid chromatography; UPLC-MS/MS: ultra-performance liquid chromatography tandem mass-spectrometry; HILIC: hydrophilic interaction chromatography; m/z_RT: mass to charge ratio _ retention time; PCA: principal component analysis; OPLS-DA: orthogonal projection to latent structures discriminant analysis; CV: coefficient of variation; BSH: bile salt hydrolase; LPE: lysophosphatidylethanolamine; LPC: lysophosphatidylcholine; TAUT: taurine transporter

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Abstract

Pregnancy is associated with progressive hypercholanemia, hypercholesterolemia and hypertriglyceridemia, which can result in metabolic disease in susceptible women. Gut signals modify hepatic homeostatic pathways, linking intestinal content to metabolic activity. We sought to identify whether enteric endocrine signals contribute to raised serum bile acids observed in human and murine pregnancies, by measuring fibroblast growth factor (FGF)19/15 protein and mRNA levels,

and 7α-hydroxy-4-cholesten-3-one. Terminal ileal farnesoid X receptor(FXR)-mediated gene expression and apical sodium bile acid transporter (ASBT) protein concentration were measured by qPCR and western blotting. Shotgun whole genome sequencing and UPLC-MS were used to determine the cecal microbiome and metabonome. Targeted and untargeted pathway analyses were performed to predict the systemic effects of the altered metagenome and metabolite profiles. Dietary cholic acid supplementation was used to determine whether the observed alterations could be overcome by intestinal bile acids functioning as FXR agonists.

Human and murine pregnancy were associated with reduced intestinal FXR signaling, with lower FGF19/15 and resultant increased hepatic bile acid synthesis. Terminal ileal ASBT protein was reduced in murine pregnancy. Cecal bile acid conjugation was reduced in pregnancy due to elevated bile salt hydrolase-producing *Bacteroidetes*. Cholic acid supplementation induced intestinal FXR signaling, which was not abrogated by pregnancy, with strikingly similar changes to the microbiota and metabonome as identified in pregnancy.

Conclusion: the altered intestinal microbiota of pregnancy enhance bile acid deconjugation, reducing ileal bile acid uptake and lowering FXR induction in enterocytes. This exacerbates the effects mediated by reduced bile acid uptake transporters in pregnancy. Thus, in pregnant women and mice, there is reduced FGF19/15-mediated hepatic repression of hepatic bile acid synthesis, resulting in hypercholanemia.

In normal human pregnancy, there is a change in metabolism with advancing gestation that results in elevated serum bile acids(1), hypercholesterolemia and hypertriglyceridemia(2) in the third trimester. These gestational alterations in lipid metabolism are essential to ensure nutrient provision for the growing fetus, and are associated with altered expression of key genes in pathways that control bile acid and lipid homeostasis. Serum and hepatocyte bile acid concentrations are elevated on day 18 of murine pregnancy(3, 4), likely secondary to gestational increases in 17β-estradiol(5) and sulfated progesterone metabolites(6). These endocrine changes cause reduced function of hepatic farnesoid X receptor (FXR); the sulfated progesterone metabolite, epiallopregnanolone sulfate (PM5S) is a partial agonist of FXR (7), whilst 17β-estradiol-bound estrogen receptor α directly interacts with FXR to repress downstream transcription(5). Bile acids also signal via enterocyte and enteroendocrine L-cell receptors to influence bile acid homeostasis and the release of hormones that impact gestational lipid and bile acid metabolism. Activated FXR in enterocytes dimerizes with retinoid X receptor (RXR), causing induction of rodent ileal bile acid binding protein (IBABP)(8), organic solute transporters (OSTα and OSTβ)(9), secretion of the hormone fibroblast growth factor 15 in mice, FGF19 in humans (10), and small heterodimer partner (SHP)-mediated repression of apical sodium dependent bile acid transporter (ASBT)(11). Bile acid-binding and activation of the G-protein coupled receptor TGR5, expressed by enteroendocrine cells within the gut, results in release of glucagon-like peptide 1 (GLP1)(12). Thus, luminal bile acids have a profound effect on bile acid, lipid and glucose homeostasis through interaction with different receptors in a range of tissues(13).

The composition of the gut microbiota influences the luminal concentration and conjugation of specific bile acids, and consequent expression of enzymes that control intestinal and hepatic bile acid transport and metabolism via FXR-dependent pathways (reviewed in (14)). In brief, when compared with germ-free mice, conventionally-reared animals had higher serum and fecal bile acid concentrations, more deconjugated bile acids in the distal gut, and a lower proportion of taurine-conjugated bile acids in the proximal and distal gut(15) and other tissues including the kidney and

heart(16). The potency of bile acid species as FXR agonists differs(17), chenodeoxycholic acid, cholic acid and their taurine conjugates are FXR agonists, whilst the taurine-conjugated α and β muricholic acids (MCA) are FXR antagonists(15). Furthermore, alterations in the luminal concentration of specific bile acids will affect the abundance of particular microbes within the gut lumen; for example, *Bilophila wadsworthia* grows preferentially in the presence of high bile acid concentrations(18), whilst deoxycholic acid and chenodeoxycholic acid are toxic to the growth of *Bifidobacterium breve, Blautia coccoides* JCM 1395^{T} and *Bacteroides thetaiotaomicron* DSM 2079^{T}(19). FXR activity in the intestine has been shown to regulate bacterial growth: administration of the synthetic FXR agonist, GW4064, after bile duct ligation (when bacterial overgrowth is common) reduced both aerobic and anaerobic bacteria in the ileum and cecum; this effect did not occur in $Fxr^{-/-}$ mice(20).

Studies of pregnancy typically demonstrate alterations in the intestinal gut microbiota through gestation; two human studies reported marked alterations in the third trimester compared with the first(21,22), with resultant metabolic changes when third trimester feces was used to colonise germ-free mice(21). A longitudinal study in mice demonstrated that changes to the gut microbiota occurred early in gestation(23), however, a longitudinal human study of 49 women did not show gestational alterations in gut bacterial composition(24).

In this study, we sought to determine whether altered enterohepatic feedback contributes to the hypercholanemia of pregnancy, including whether gestational alterations in the human gut microbiota and intestinal metabonome could contribute. We then utilized a murine model of cholic acid feeding, which has similar phenotypic differences in lipid composition and hepatic steatosis to pregnancy(3), to establish whether enterocyte exposure to an FXR ligand abrogated these gestational effects.

Experimental procedures

Human samples

The study conformed to the 1975 Declaration of Helsinki guidelines; permission was obtained from the ethics committees of Hammersmith Hospitals NHS Trust, London (08/H0707/21 and 11/LO/0396) and Sahlgrenska University Hospital, Gothenburg (Dnr 536-14). Written informed consent was received from participants prior to inclusion.

Fecal samples were obtained from 14 women with uncomplicated pregnancies and 9 non-pregnant healthy women. Women were restricted to those with spontaneously-conceived third trimester singleton pregnancies, without pregnancy complications and who had not taken antibiotics for the duration of the pregnancy. Fecal samples were frozen at -80^{o}C within 24 hours of collection.

Serum samples for analysis of FGF19 and 7α-hydroxy-4-cholesten-3-one (C4) were taken from women given a standardized diet from 18:00 the preceding day, with venepuncture performed at 8:00 (fasting) and 15:00. Participants were given fixed meals at 18:00, 08:00 and 12:00, and remained sedentary throughout. Of the participants, 14 were non-pregnant (with previously uncomplicated pregnancies), and 24 had uncomplicated pregnancies. Blood samples were obtained in Vacutainer Serum Separation Tubes, and after 30 minutes were centrifuged at 1700*g* for 10 minutes. Serum was aliquoted and stored at -80^{o}C. Serum analyses for FGF19 and C4 were performed as previously described(25), using enzyme-linked immunosorbent assay (ELISA) (FGF19 Quantikine ELISA kit, Cat. No. DF1900; R&D Systems, Minneapolis, MN), and high-performance liquid chromatography (HPLC) following solid phase extraction for C4.

Animal handling

Experiments were conducted according to the UK Animals (Scientific Procedures) Act of 1986 and with protocols approved by the King's College London Animal Studies Committee. Female C57BL/6 mice (Charles River, UK) were housed in the same room within clean facilities, with a 12-hour light cycle. Cages housed 3 mice per cage, all from the same dietary group. Mice were fed a normal chow diet (RM3 control diet) *ad libitum* until mating, at which time half the mice had a diet supplemented with 0.5% cholic acid (LBS Biotech, UK) until delivery. Mice were sacrificed on day 0 or day 18 of pregnancy, resulting in n=6-8 per group. Separate cohorts of C57BL/6 mice were sacrificed at increasing gestational ages (day 2, 7, 10, 14, 18, n=6-7 per timepoint), as previously described(26).

At sacrifice, murine ceca, ileum, duodenum, gall bladder and liver were harvested and snap frozen on dry ice and subsequently stored at -80^{o}C. Whilst frozen, ceca were longitudinally split, and content dissected from overlying intestinal tissue.

DNA extraction

DNA was extracted from cecal content using Qiagen Tissuelyser II bead beating and QiaAMP Fast DNA Stool Mini Kit (Qiagen, UK), according to manufacturer's instructions.

Metagenomics analysis

The 29 murine samples of extracted DNA were treated with PowerClean DNA Clean-Up Kit (Mo Bio, US), as per manufacturer's instructions. The samples were sequenced by The Genome Analysis Centre (TGAC, UK) using an Illumina HiSeq 2500 platform (Illumina, US) in paired-end mode, with typical read lengths 85-150bp. The samples were checked using FastQC(27) to assure the global

quality of the sequencing and only one sample (non-pregnant, chow fed) was removed thereafter, leaving n=6-8 per group.

An in-house metagenomics analysis pipeline was developed as previously described(28) introducing the removal of the repeat masking step and the inclusion of the assembly step using IDBA-UD software(29) (Supplementary Figure 1). Methods for this are included in the Supplementary Materials.

Metataxonomic analysis (16S rRNA gene sequencing)

Metataxonomics was performed by Research and Testing, Texas. Taxonomic identification of sequences was performed using QIIME software referencing the GreenGenes library, with statistical results analysis using STAMP and R. The p-values were adjusted for multiple comparisons using the Benjamini-Hochberg method in R programming software.

Metabolic profiling

Aqueous and organic extractions were performed for cecum, cecal content, gall bladder and liver samples as detailed in Supporting Information. These were assessed with reversed phase-ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) lipid profiling of organic extracts, hydrophilic interaction chromatography (HILIC) UPLC-MS/MS profiling of aqueous extracts and bile acid UPLC-MS/MS profiling of combined aqueous and organic extracts.

Data pre-processing and statistical analysis of metabolic profiling

The MS raw data were converted to netCDF format using the DataBridge tool implemented in MassLynxTM software (Waters Corporation, US). The data were processed using the XCMS package in R (https://www.r-project.org/), and an output table was obtained comprising pairs of m/z_RT (mass to charge ratio _ retention time) and intensity values of the detected metabolite features in each sample. The dataset was normalized to total area normalization. Multivariate data analysis was performed using the SIMCA package (v.13.0.2, Umetrics, Sweden). Principal component analysis (PCA) and orthogonal projection to latent structures discriminant analysis (OPLS-DA) were used to examine UPLC-MS data in a multivariate setting. Prior to model fitting, features were subjected to Pareto scaling. Two-tailed t-test assuming unequal variance, and coefficient of variation % (CV %) were calculated in Microsoft Office Excel 2007. The p-values were adjusted for multiple comparisons using the Benjamini-Hochberg method in R programming software.

Metabolite Assignment

Metabolite identification by MS was conducted by matching accurate m/z measurements of detected chromatographic peaks to theoretical values from in-house databases and on-line databases (Human metabolite database (HMDB, http://www.hmdb.ca/), KEGG (http://www.genome.jp/kegg/ligand.html), LIPID MAPS (http://www.lipidmaps.org/tools/index.html), and METLIN (http://metlin.scripps.edu/)). Tandem MS fragmentation patterns were obtained for further structural elucidation. Assignment was confirmed by the comparison of retention times and MS/MS data with authentic standards (details available on request).

Tissue mRNA expression

Total RNA from duodenum, distal ileum and livers of mice was extracted and quantified with real-time PCR, as described in the Supplementary Methods.

Tissue protein expression

Distal ileal ASBT protein levels were measured using western blotting, as described in the Supplementary Methods.

Results

Pregnancy is associated with reduced enterohepatic bile acid-FXR mediated signaling

Fasting and peak FGF19 and C4 levels were measured in the serum of women taking a standardized diet for 24 hours. Women in the third trimester of pregnancy had reduced peak FGF19, demonstrating reduced intestinal FXR signaling and enterohepatic feedback (Figure 1a). This reduction was consistent with the increased gestational hepatic bile acid synthesis demonstrated by elevated fasting serum concentrations of C4 (Figure 1b). Lowered FGF19 levels were not explained by intestinal FXR inhibition by epiallopregnanolone sulfate (PM5S), which increases in normal pregnancy and can impair hepatic FXR induction, as CDCA-induced FXR induction was not affected by co-treatment with PM5S in terminal ileal explants (Supplementary Figure 2). As bile acid species have different potencies as FXR agonists, fecal bile acids were measured. There were minimal differences between non-pregnant women and those in the third trimester of uncomplicated pregnancies (Figure 1c). However, the majority of fecal bile acids will be deconjugated by colonic bacterial activity by the time they are excreted in feces, and therefore this result is unlikely to reflect the bile acid content of the terminal ileum, the site of maximal enterocyte FXR expression. To address this issue, cecal contents were collected from C57BL/6 pregnant mice.

Murine pregnancy is characterized by impaired intestinal FXR signaling, with altered intestinal bile acid conjugation

To determine the influence of pregnancy on intestinal tissues, terminal ileal and cecal samples from C57BL/6 mice were examined. Terminal ileal FXR induction of FGF15 and SHP was reduced on day 18 of murine pregnancy (Figure 2a). Whilst expression of ASBT mRNA did not differ significantly in pregnancy, despite its usual repression by SHP (Figure 2a), ASBT protein levels were significantly reduced (Figure 2b). Consistent with the human explant models, PM5S did not repress terminal ileal FXR induction in mice that received PM5S gavage compared with vehicle (Supplementary Figure 3). Given the gestational reduction in terminal ileal FXR induction, we determined hepatic mRNA expression of genes of relevance to bile acid homeostasis, to establish whether these intestinal signals contribute to hypercholanemia (Supplementary Figure 4a). There were gestational reductions in mRNA expression of Fxr target genes as has been previously described(4). Intriguingly, there was reduced transcription of *Cyp7a1* at day 18 of pregnancy, a result that is not consistent with reduced bile acid synthesis. As murine pregnancy is associated with hypercholanaemia with advancing gestation which could repress *Cyp7a1* at the hepatocyte(3), we quantified hepatic *Cyp7a1* expression in mice throughout pregnancy, and demonstrated a significant increase at day 7 of pregnancy, which fell by day 14 (Supplementary Figure 4b).

Targeted UPLC-MS/MS evaluation of cecal content demonstrated lower conjugated and sulfated bile acids in pregnancy (Figure 2c,d, Supplementary Table 2), with proportionately more secondary (deoxycholic acid, ω-muricholic acid) than primary bile acids (cholic acid, α-muricholic acid, β-muricholic acid) than in age-matched non-pregnant mice (Figure 2e). The reduction in conjugated bile acids in the cecum in pregnancy did not result from lower hepatic bile acid conjugation, as the

bile acid profiles of liver and gall bladder did not reveal altered conjugation proportions in pregnancy (Supplementary Figure 5).

The cecal microbiome of murine pregnancy has elevated *Bacteroidetes*, with enhanced bile salt hydrolase genes and bile salt deconjugation

As the cecal bile acid composition was likely altered by the intestinal bacterial modification, whole shotgun genome metagenomic sequencing was used to characterize the effect of pregnancy on the murine cecal microbiota. Pregnancy was associated with a higher ratio of *Bacteroidetes* to *Firmicutes*, with increased richness and diversity (Figure 3a, b, c, Supplementary Table 3). Targeted metagenomics confirmed the pregnant microbiome encoded bile salt hydrolase (*Bsh*), which deconjugates bile acids; further analysis revealed this to be encoded by the genome of bacteria of the *Bacteroidetes* phylum (Figure 3d). Similarly, the microbiome of pregnant mice included genes for aryl sulfatases, also *Bacteroidetes*-derived, which were not present outside pregnancy (Figure 3e). 7-α-dehydroxylase sequences from the Uniprot library were not identified in the microbiome of either group. However, none of these sequences originates from *Bacteroidetes*, which are likely to have contributed to the production of secondary bile acids.

The gestational murine cecal metabonome markedly differs from non-pregnant mice, with lower taurine secondary to its bacterial metabolism

Given the multiple metabolic actions of intestinal bacteria, untargeted UPLC-MS/MS was performed to identify metabolites that were significantly altered in the cecal content of pregnant mice (Figure 4a, b, summary of model characteristics in Supplementary Table 4). Taurine was significantly reduced in pregnancy, despite the increased deconjugation inferred by cecal bile acid profiles and bacterial BSH. The cecal microbiota in pregnancy was enriched with sulfur-utilizing bacteria, such as

the *Proteobacteria Bilophila* (Supplementary Table 3); these likely utilized the liberated taurine from *Bacteroidetes* bile salt hydrolase bile acid deconjugation, or sulfur following aryl sulfatase activity, as a growth substrate. Untargeted metabonomics did not detect any differences in known FXR ligands, although the retinoid X receptor (RXR) ligands, docosahexaenoic acid, arachidonic acid and docosapentaenoic acid (30) were increased in pregnancy.

Cholic acid feeding of mice reversed the gestational impairment of FXR-mediated enterohepatic feedback

Given that these results suggest that the impairment in FXR-mediated enterohepatic feedback in pregnancy is due to altered intestinal bile acid species availability, with reduced terminal ileal uptake due to the lower ASBT affinity of unconjugated bile acids, we sought to identify whether this could be overcome with an increased intestinal bile acid load. A diet supplemented with 0.5% cholic acid was fed to pregnant C57BL/6 mice and to age-matched non-pregnant mice. Cholic acid-supplemented mice had increased terminal ileal FXR induction, with elevated FGF15 mRNA expression (Figure 5a). Similarly, the cholic acid- fed mice did not have the suppression of SHP (Figure 5b) that is seen in normal gestation. Supplementation with cholic acid altered the cecal bile acid composition, with increased cholic acid and its bacterially-derived metabolite, deoxycholic acid (Figure 5c,d). Cholic acid-fed mice had very low cecal muricholic acid species; these are dependent upon murine hepatic synthesis, confirming that cholic acid supplementation overcame the gestational impairment in FXR-mediated enterohepatic feedback.

Cholic acid supplementation had similar effects on the cecal metabonome to pregnancy

To support the hypothesis that intestinal induction of FXR resulted from the altered bile acid profile, untargeted metabonomics was performed. Cholic acid supplementation resulted in very similar metabolite profiles to pregnancy (Figures 4,6).

The gestational alterations to the cecal microbiota are enhanced in cholic acid-supplemented mice

Cholic acid supplementation enriched the gut microbiota with *Bacteroidetes* and *Proteobacteria*, with reduced *Firmicutes*, similar to the changes seen in pregnancy (Figure 7), although the increased richness and diversity was only demonstrated in the pregnant cholic acid-fed mice. Whilst pregnancy ameliorated the cholic acid-feeding enhancement of *Sutterella* and *Prevotella*, the pregnancy-associated increase of *Dehalobacterium* was seen irrespective of cholic acid supplementation. Additionally, cholic acid supplementation enhanced bacteria encoding genes for BSH expression. Ordination methods of comparing the cecal metabonome and microbiota demonstrated that cholic acid feeding enhances the gestational changes observed (Figure 8a,b). A heat map correlating individual microbial and metabolite levels for the different murine groups demonstrated that the alterations observed were particularly similar for both gestational and cholic acid-feeding effects (Figure 8c), demonstrating the critical interrelationship between the gut microbial profile and metabolite composition. In both groups, the taurine-conjugated bile acids were negatively correlated with the microbes that became more abundant in pregnancy (*Bacteroides, Odoribacter, Prevotella, Bilophila* and *Desulfovibrio*), and they had a positive correlation with those that were reduced (*Parabacteroides* and *Bulleidia*). Taurine showed similar correlations with the microbiota to the taurine-conjugated bile acids, and there was an increased abundance of microbes that are known to utilize taurine for energy in the cecal content of pregnant and cholic acid-fed animals, such as *Bilophila* and *Desulfovibrio* spp. Of the phospholipids, lysophosphatidylethanolamines (LPE) and lysophosphatidylcholines (LPC) positively correlated with the microbes that were increased in

pregnancy and cholic acid feeding (e.g. *Bacteroides, Oridobacter, Prevotella, Bilophila* and *Desulfovibrio*), and negatively correlated with those that were reduced (*Bulleidia*, *Coprococcus* and *Parabacteroides*). The only exception was LPE (17:1), which had the reverse correlation. For pregnant cholic acid-fed mice, the associations between metabolites and microbiota more closely reflected those seen for cholic acid-fed mice than pregnancy alone (e.g. for inosine, docosenoic acid, tetracosanoic acid, diacylglycerol, and the bile acids), although this was not universal (e.g. taurine).

Using the metagenomic functional analysis and metabolite results, the effects of pregnancy, cholic acid feeding and cholic acid feeding in pregnancy on metabolic pathways of the mice (host) and microbiota (colonizer) were determined. Results were strikingly similar for the effects of pregnancy and cholic acid feeding, especially with respect to the predicted induction of host hepatotoxic pathways (Supplementary Table 5). Overlap in the pregnancy/ cholic acid-fed comparators is illustrated in the significantly altered toxicology functions (Supplementary Figure 6), diseases and biofunction pathways and canonical pathways (Supplementary Figures 7,8). Specifically, for normal pregnancy, the ten most significantly altered toxicological functions included six functions implicated in liver damage or liver steatosis, and eight such pathways were altered in cholic acid-fed animals, the majority of which were the same as in normal gestation (Supplementary Figure 6). Pathway analyses further revealed the cecal microbiome of pregnancy and cholic acid feeding in mice to have significantly increased abundance of genes encoding components of the glycine cleavage system (Supplementary Figure 9), consistent with utilization of glycine as an additional energy source, likely available following the additional deconjugation of glycine-conjugated bile acids.

Discussion

Our results demonstrate that normal pregnancy is associated with impaired FXR-mediated enterohepatic feedback and elevated hepatic bile acid synthesis, which is consistent with the elevation in serum primary bile acids (CA>CDCA) observed in human pregnancy(1). We concluded that this impairment results from an altered intestinal bile acid composition secondary to gestational changes in the gut microbiota, with enhanced *Bacteroidetes*-mediated bile acid deconjugation in combination with reduced terminal ileal bile acid uptake in pregnancy secondary to lower ASBT protein levels. Direct signaling from the gut bacteria to FXR, or other alterations in the gut metabonome are unlikely to cause this impairment in enterohepatic feedback, as cholic acid dietary supplementation resulted in a very similar gut microbiome and metabolome to pregnancy, yet resulted in induction of enterohepatic feedback. Pregnancy and cholic acid supplemented mice had increased cecal ratios of *Bacteroidetes* to *Firmicutes*, with additional enrichment of sulfur-reducing bacteria. Bile salt hydrolase was exclusively detected in the genome of the *Bacteroidetes* present in these animals, and these mice had proportionately more cecal unconjugated bile acids.

The elevation in serum bile acids in pregnancy has previously been attributed to reduced enterohepatic cycling(31), consistent with reduced FXR function and *Bsep* induction(4,7); our demonstration of elevated fasting C4, in particular, supports altered regulation of bile acid synthesis in pregnancy, likely secondary to reduced FXR function and resultant lowering of SHP-mediated impairment of CYP7A1. Whilst we demonstrated reduced Cyp7a1 mRNA expression at day 18, the increased transcription at day 7 is consistent with hepatic FXR inhibition; protein quantification and activity assays would be required to confirm this mechanism of increased gestational bile acid synthesis. Herein, we provide an additional explanation, that increased hepatic bile acid synthesis is secondary to reduced enterohepatic feedback, which is consistent with the previous murine study of Moscowitz and colleagues(32). A limitation of our study was that we did not measure the complete

bile acid pool size and total bile acid excretion, to confirm that increased bile acid synthesis was not compensatory to increased fecal bile acid loss.

Alterations in the intestinal bile acid composition, resulting from bacterial bile acid modification, could impact enterocyte FXR induction in two ways: by impairing bile acid uptake by the enterocyte, and/or by changing the proportion of agonistic bile acid ligands compared the antagonists. ASBT-binding of bile acids is thought to be the rate-limiting step in the uptake of bile acids from the lumen to terminal ileal enterocytes, and ASBT preferentially binds conjugated bile acids (reviewed in (33)). Thus, we hypothesize that the proportionate reduction in conjugated bile acids in pregnancy, secondary to bacterial activity, reduces the amount of bile acids absorbed in the terminal ileum, the predominant site of FGF19/15 synthesis. Whilst there was no reduction in ASBT mRNA induction in pregnancy, ASBT protein levels were reduced in the distal ileum, which likely contributed further to the reduced intestinal FXR induction.

Established ligand potencies of the bile acids present in the pregnant cecal content are consistent with altered FXR induction as a consequence of the different balance of agonistic bile acids, such as chenodeoxycholic acid and deoxycholic acid species(17), with antagonistic bile acids, such as the tauromuricholic acids(15). The profile of cecal bile acids in pregnant mice did not suggest these mice would have associated impairment of intestinal FXR function, as there were significant reductions in the antagonistic species.

The similarities in the intestinal metabonome and microbiota between the pregnant and cholic acid fed mice were surprising, given the markedly different bile acid loads to the intestine. Our findings are in contrast to those of cholic acid dietary supplementation in rats, where increased cecal cholic

acid and deoxycholic acid induced expansion of the Firmicutes, particularly the Clostridia class (34). This may have been due to the increased lysophospholipids (LPE/LPC) in the cecal content; these lysophospholipids are bactericidal to gram negative bacteria, particularly anaerobes such as Clostridia (35, 36). Given the structural similarity of steroid hormones to bile acids, the gestational increases in estrogens, progestogens and corticosteroids, present in bile, (reviewed by Begley and colleagues(37)) may provide similar bacterial selection pressures to those of bile acid feeding. Indeed, steroid hormones, such as 17α-hydroxyprogesterone, estradiol and progesterone, are both inhibitory and stimulatory to growth of different bacteria (38-40).

In summary, we have demonstrated alterations in the intestinal microbiome and metabolome in pregnancy that could impair enterohepatic feedback on bile acid synthesis and contribute to the hypercholanaemia of pregnancy. Particularly as the secondary bile acids, DCA and LCA, are also potent ligands for the TGR5 receptor at enteroendocrine L cells, the altered microbial modification of bile acids in pregnancy may explain the increasing levels of GLP1 with advancing gestation(41). Future studies are needed to establish precisely how gestational signals are capable of dramatically impacting the gut microbiome during pregnancy, and whether these gestational signals can be manipulated to enhance beneficial metabolic changes for the pregnancy. This understanding will be of importance for the treatment of such gestational metabolic disorders as intrahepatic cholestasis of pregnancy and gestational diabetes mellitus.

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Author names in bold designate shared co-first authorship

Figure legends

Figure 1. Human pregnancy reduces enterohepatic feedback, without alteration in fecal bile acids

(A) Fibroblast growth factor 19 (FGF19) and **(B)** 7α-hydroxy-4-cholesten-3-one (C4) in the serum of 14 non-pregnant women and 24 pregnant women with uncomplicated pregnancies whilst fasting (08:00) and three hours post-prandial (15:00), following a standardized diet. Boxes show interquartile range (IQR) with whiskers at 1.5 IQR. Groups compared with 2-way ANOVA and Tukey testing for multiple comparisons.

(C) Bile acid profile of fecal samples from women outside of pregnancy (dark grey bars, n=9), and with uncomplicated pregnancies (light grey bars, n=14). Bars show mean ± SEM. Significance determined by 2-way ANOVA with Tukey's multiple comparison test. CA: cholic acid, CDCA: chenodeoxycholic acid, DCA: deoxycholic acid, LCA: lithocholic acid, UDCA: ursodeoxycholic acid, MCA: muricholic acid, HCA: hyocholic acid, HDCA: hyodeoxycholic acid, TCA: taurocholic acid, TCDCA: taurochenodeoxycholic acid, TDCA: taurodeoxycholic acid, GCA: glycocholic acid, GCDCA: glycochenodeoxycholic acid, GDCA: glycodeoxycholic acid.

Figure 2. Murine pregnancy is characterised by reduced enterohepatic feedback with an altered cecal bile acid profile.

(A) Expression levels of fibroblast growth factor 15 (FGF15), small heterodimer partner (SHP) and apical sodium bile acid transporter (ASBT) mRNA in murine distal ileum of non-pregnant and pregnant (day 18) mice, assessed by multiple measures of ANOVA with Tukey post hoc testing. Boxes show IQR with whiskers at 1.5 IQR, N=6-8.

(B) Protein levels of ASBT in murine distal ileum determined by western blotting for non-pregnant and pregnant mice (n=8 and n=5), normalised to βactin. Groups compared with Mann-Whitney test.

(C) Ratio of conjugated to unconjugated bile acids in the cecal content of non-pregnant and pregnant mice. Boxes show IQR with whiskers at 1.5 IQR, N=6-8.

(D) Distribution of cecal bile acids by conjugation in the cecal content of non-pregnant and pregnant mice. N=6-8.

(E) Relative quantification of individual bile acids present in cecal content of non-pregnant and pregnant mice. Assessed by Student's t test, with Benjamini-Hochberg correction, results show mean +SEM, N=6-8. CA: cholic acid, DCA: deoxycholic acid, CDCA: chenodeoxycholic acid, LCA: lithocholic acid, UDCA: ursodeoxycholic acid, MCA: muricholic acid, HCA: hyocholic acid, HDCA: hyodeoxycholic acid, MDCA: murideoxycholic acid, TCA: taurocholic acid, TDCA: taurodeoxycholic acid, THDCA: taurohyodeoxycholic acid, TMCA: tauromuricholic acid, TUDCA: tauroursodeoxycholic acid.

Figure 3. Pregnancy alters the murine cecal microbiota and microbiome.

(A) Distribution of cecal microbiota by phylum level for non-pregnant and pregnant mice. N=6-8.

(B) Chao 1 richness and **(C)** Shannon evenness of microbial communities for non-pregnant and pregnant mice. Box-whisker plots demonstrate median (thick line) and interquartile range (IQR) with

whiskers at 1.5 IQR. Differences between groups determined by Kruskal Wallis univariate analysis. N=6-8 throughout.

(D) Bile salt hydrolase (*Bsh*) and **(E)** aryl sulfatase (*Ars*) gene reads in the cecal microbiome of non-pregnant and pregnant mice. Difference between groups determined by two-tailed t-test. N=6-8 throughout. ns: not significant ($p \geq 0.05$).

Figure 4. Pregnancy alters the murine cecal metabonome.

Metabolite changes in the cecal content between non-pregnant and pregnant mice. **(A)** Significance of metabolite changes, log (p-value) of t-test (two-tailed; assuming unequal variance) and **(B)** fold change for the same metabolites. p-values adjusted for multiple comparisons using the Benjamini-Hochberg method. N=6-8. Cer: ceramide, DG: diacylglycerol, HexCer: hexosylceramide, PA: phosphatidic acid, PE: phosphatidylethanolamine, PC: phosphatidylcholine, SM: sphingomyelin. N=6-8.

Figure 5. Cholic acid supplementation enhances enterohepatic feedback, and alters the cecal bile acid profile.

(A) Expression levels of fibroblast growth factor 15 (FGF15), and **(B)** small heterodimer partner (SHP) mRNA in murine distal ileum of non-pregnant chow and cholic acid fed mice and pregnant cholic acid fed mice, assessed by multiple measures of ANOVA with Tukey post hoc testing. Boxes show IQR with whiskers at 1.5 IQR, N=6-8.

(C) Distribution of cecal bile acids by conjugation in the cecal content of non-pregnant chow and cholic acid fed and pregnant cholic acid fed mice. N=6-8.

(D) Relative quantification of individual bile acids present in cecal content of non-pregnant chow and cholic acid fed mice and pregnant cholic acid fed mice. Assessed by two-tailed t test, with Benjamini-Hochberg correction, bars show mean+SEM, N=6-8. Significance determined by $p<0.05$; a: non-pregnant chow vs non-pregnant cholic acid fed mice; b: non-pregnant cholic acid fed vs pregnant cholic acid fed mice; c: non-pregnant chow fed vs pregnant cholic acid fed mice. CA: cholic acid, DCA: deoxycholic acid, CDCA: chenodeoxycholic acid, LCA: lithocholic acid, UDCA: ursodeoxycholic acid, MCA: muricholic acid, HCA: hyocholic acid, HDCA: hyodeoxycholic acid, MDCA: murideoxycholic acid, TCA: taurocholic acid, TDCA: taurodeoxycholic acid, THDCA: taurohyodeoxycholic acid, TMCA: tauromuricholic acid, TUDCA: tauroursodeoxycholic acid.

Figure 6. Cholic acid dietary supplementation alters the murine cecal metabonome.

Metabolite changes in the cecal content between, from left to right, non-pregnant chow and non-pregnant cholic acid fed mice, and non-pregnant cholic acid and pregnant cholic acid fed mice. **(A)** Significance of metabolite changes, log (p-value) of t-test (two-tailed; assuming unequal variance) and **(B)** fold change for the same metabolites. p-values adjusted for multiple comparisons using the Benjamini-Hochberg method. N=6-8. Cer: ceramide, DG: diacylglycerol, HexCer: hexosylceramide, PA: phosphatidic acid, PE: phosphatidylethanolamine, PC: phosphatidylcholine, SM: sphingomyelin. N=6-8.

Figure 7. Cholic acid dietary supplementation alters the murine cecal microbiota in similar ways to pregnancy

(A) Distribution of cecal microbiota by phylum level for non-pregnant chow and cholic acid fed mice, and pregnant cholic acid fed mice. N=6-8.

(B) Bile salt hydrolase (*Bsh*) and **(C)** aryl sulfatase (*Ars*) gene reads in the cecal microbiome of non-pregnant and pregnant mice. Box-whisker plots demonstrate median (thick line) and interquartile range, with whiskers at 1.5 IQR. Differences between groups determined by one-way ANOVA with Tukey's multiple comparison test. N=6-8.

(C) Chao 1 richness and **(D)** Shannon evenness of microbial communities for non-pregnant and pregnant mice. Box-whisker plots demonstrate median (thick line) and interquartile range, with whiskers at 1.5 IQR. Differences between groups determined by Kruskal Wallis univariate analysis. N=6-8 throughout.

(E) Taxonomic tree of microbes identified at genus level found to significantly differ after separate comparisons of non-pregnant chow vs pregnant chow-fed (grey), non-pregnant chow vs non-pregnant cholic acid-supplemented diet (orange), pregnant chow vs pregnant cholic acid supplemented diet (green) and non-pregnant cholic acid vs pregnant cholic acid-supplemented diets (purple). Arrow direction indicates relative abundancy in the second of the comparator groups. Significance defined as $p<0.05$ by White's non-parametric t test with Benjamini-Hochberg correction.

Figure 8. Comparison between metabonome and microbiome of mouse cecal content by diet and pregnancy status

(A) Scores plot of principal components analysis (PCA) of the cecal content extracts generated from the UPLC-MS analysis (dark blue, non-pregnant chow-fed; light blue, pregnant chow-fed; red, non-pregnant cholic acid supplemented diet; yellow, pregnant cholic acid supplemented diet). PC1 explains 27% variance of the dataset and PC2 explains 13.6%

(B) Scores plot of principal coordinates analysis (PCoA) of the cecal content from the microbiome weighted Unifrac analysis (dark blue, non-pregnant chow-fed; light blue, pregnant chow-fed; red, non-pregnant cholic acid supplemented diet; yellow, pregnant cholic acid supplemented diet). PC1 explains 64% variance of the dataset and PC2 explains 12.6%

(C) Correlation matrix (Spearman correlation) of metabolites (y axis) and microbes (x axis) found to be significantly different after separate comparisons of non-pregnant chow vs pregnant chow-fed, non-pregnant chow vs non-pregnant cholic acid-fed, and pregnant chow vs pregnant cholic acid-fed mice. N=6-8 throughout.

Figure 1

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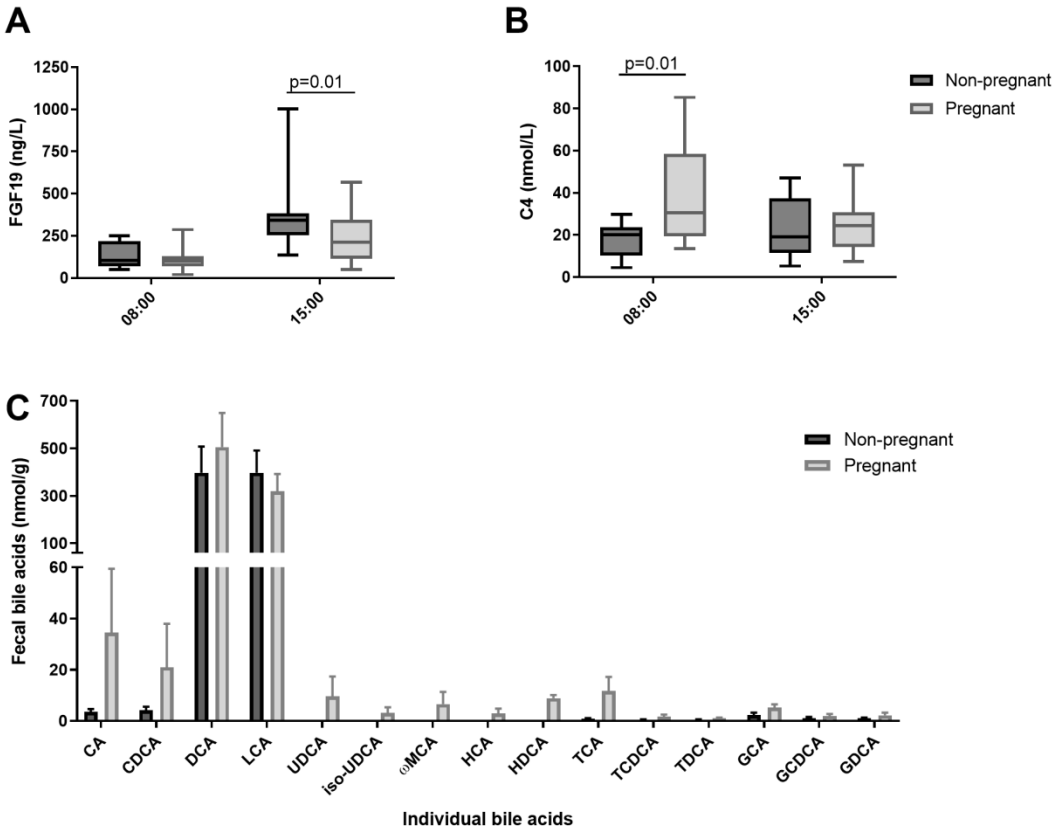

Figure 2

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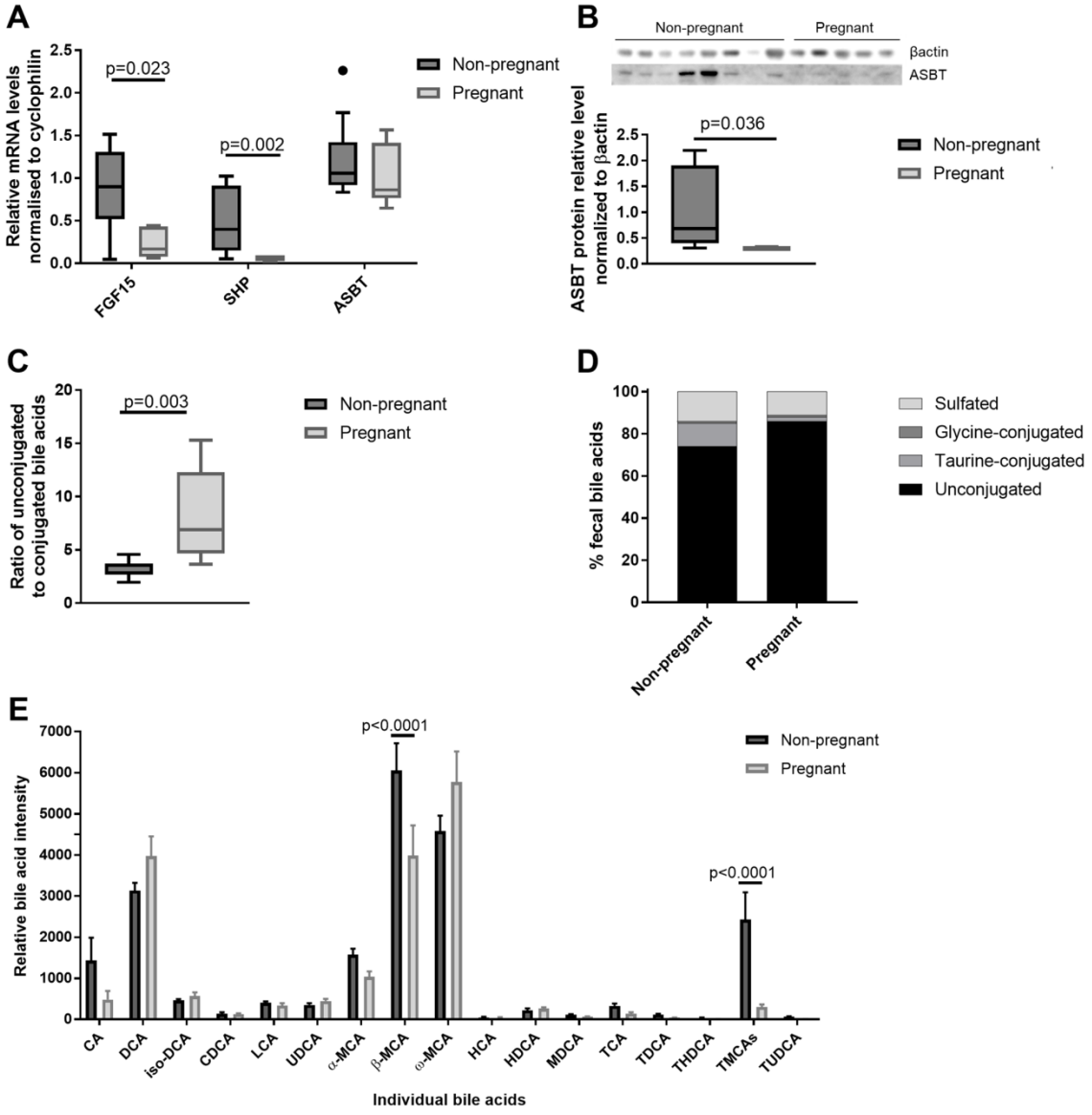

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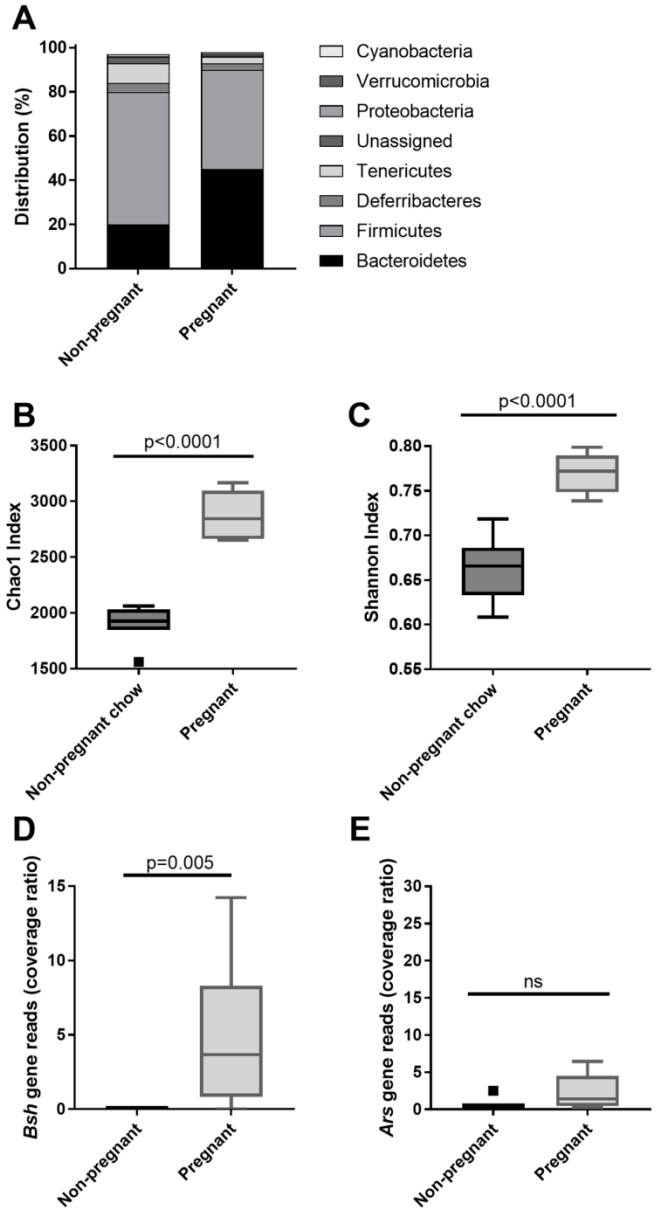

Figure 4

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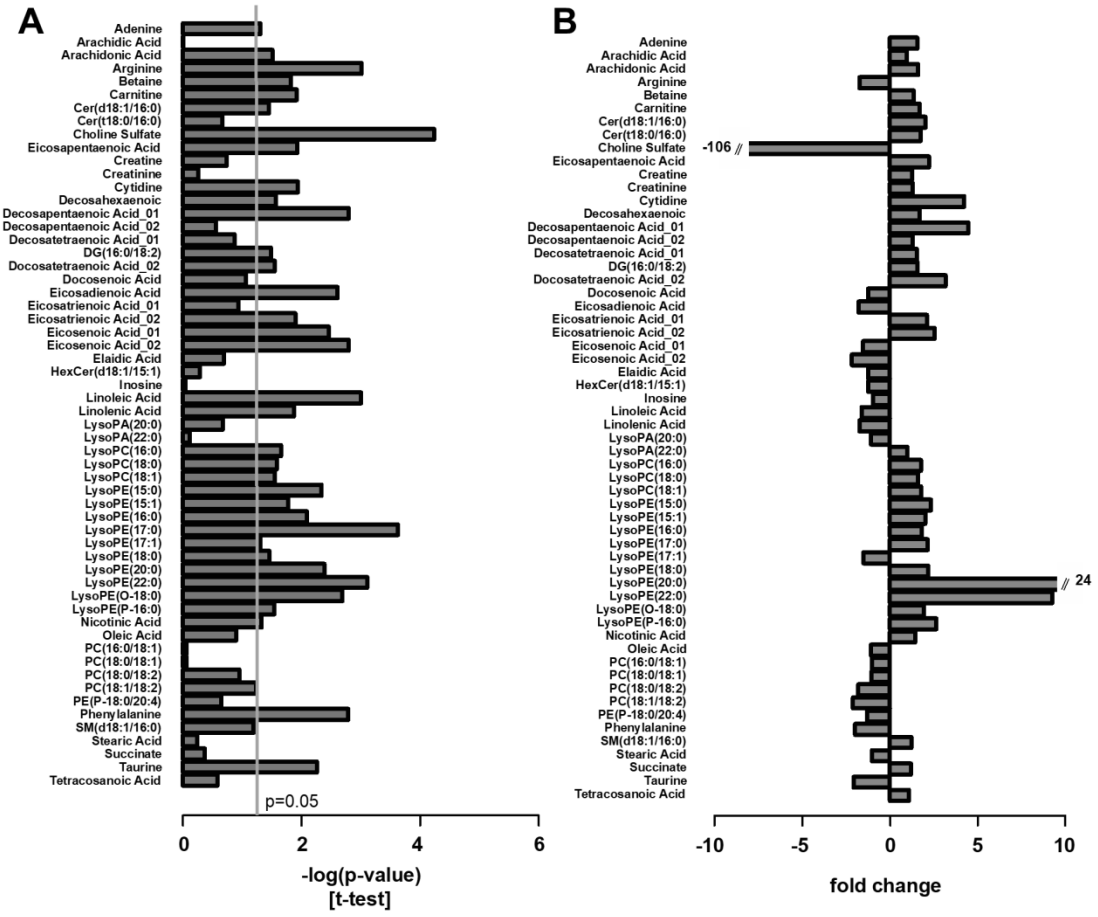

Figure 5

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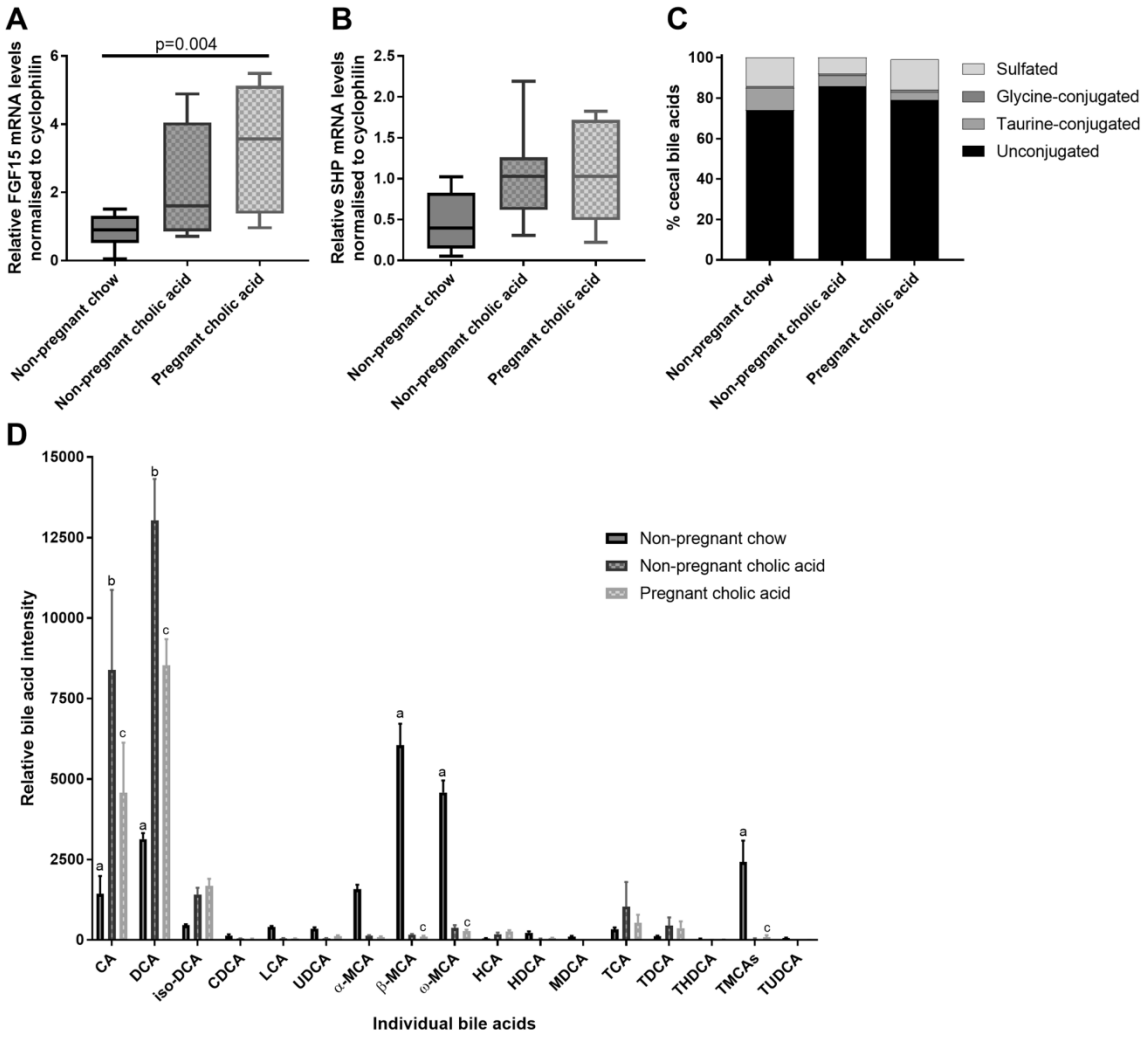

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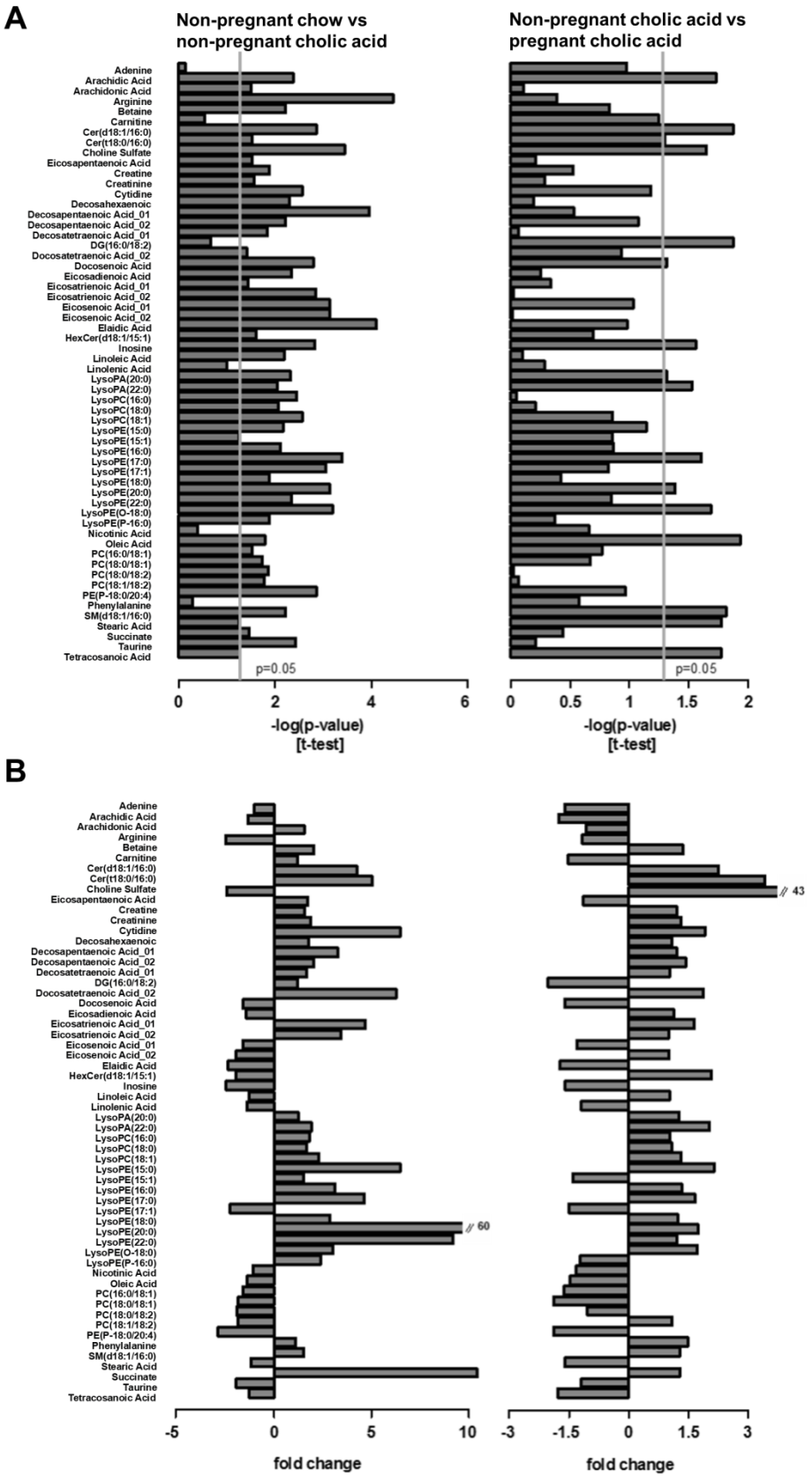

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Figure 7

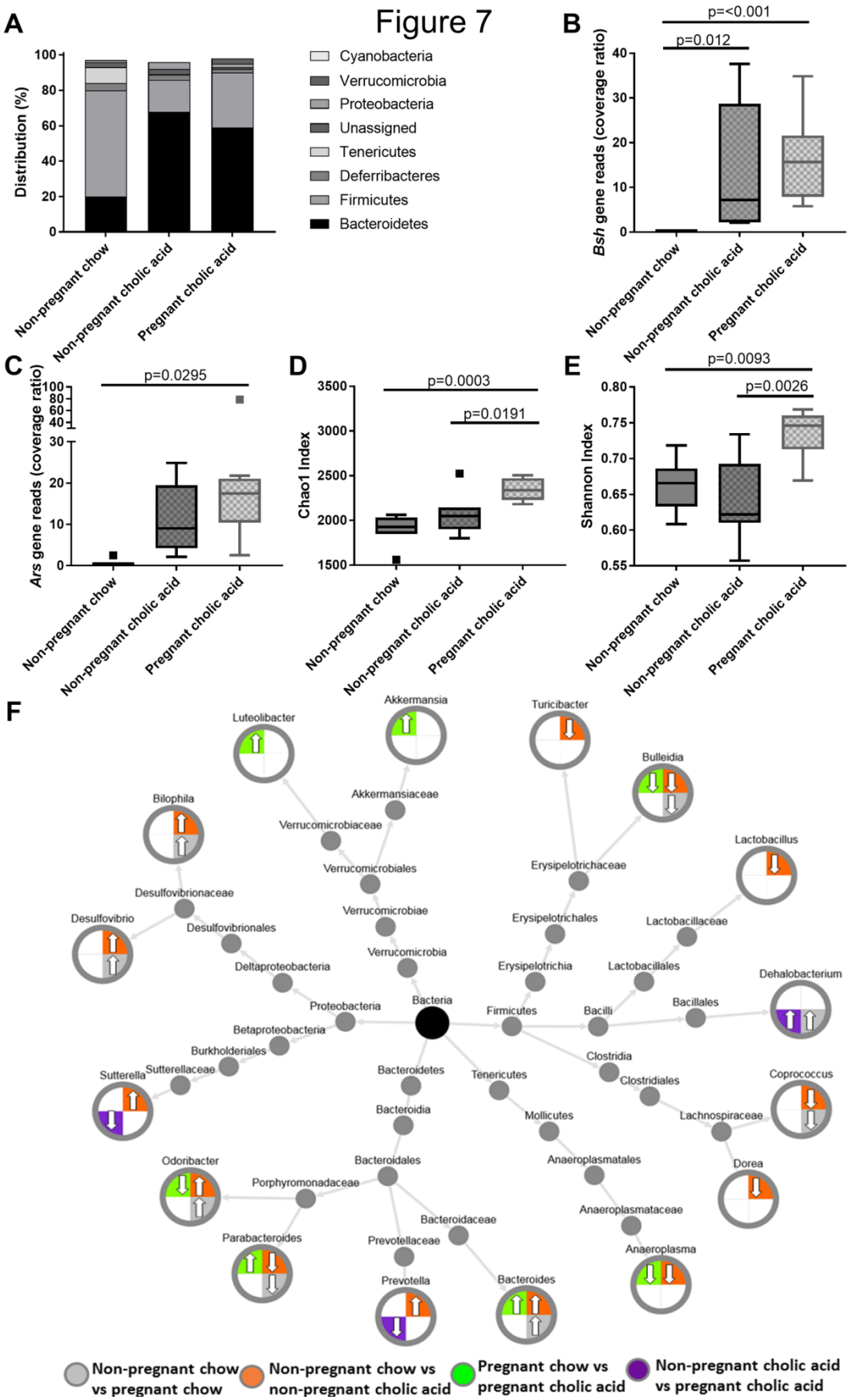

Figure 8